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Aberration of chromosomes 8 and 11 in bladder cancer as detected by fluorescence in situ hybridization

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Abstract Although a bladder cancer-specific abnormality in chromosomes or genes has not been reported, chromosomal regions that tend to become abnormal have been recognized. In this study, we investigated abnormalities in chromosomes 8 and 11. There were 27 patients with bladder cancer, 16 males and 11 females, who participated in this study. Abnormalities in chromosomes 8 and 11 were investigated by the fluorescence in situ hybridization (FISH) method. Probes used in this study were chromosome 8α-satellite and chromosome 11α-satellite (Oncor Co.). Of 27 cases, 15 cases were positive for chromosome 8 (55.6%) and ten cases were positive for chromosome 11 (37.0%). Since the FISH method detects chromosomal abnormality by the number of signals generated in cancer cells, this method is objective and simple and thus may be applicable in clinical practice.

Key words Bladder cancer · FISH method · Chromosomal abnormality

Introduction

Cytodiagnosis has been used for screening and diagnosis of malignant tumors, monitoring the course of cancer therapy and early discovery of recurrence as an established diagnostic technique. In bladder cancer, cytodiagnosis can use deciduated cells in spontaneous urine as a sample, and thus is less stressful to patients. Therefore, cytodiagnosis is frequently incorporated as a routine test applied to outpatients consulting the urologic department. In cytodiagnosis, Papanicolaou-stained preparations are macroscopically observed for the morphology of cell mass and individual cells and cancer cells are diagnosed using the morphological characteristics of cancer cells based on experience [24, 27]. Of these cases 30–40% were found to be positive; in cases showing a low degree of atypism, around 10% were positive [7, 8, 17, 23, 30]. Since these diagnostic indices are based on the degree of atypism such as size and nonuniformity of nuclei and cells, nuclear-cytoplasmic ratio (N/C) and agglutination and distribution of nuclear chromatin, it is impossible to avoid subjective factors such as the experience and knowledge of the examiners. Furthermore some cancer cells exhibit intermediate features between benign and malignant tumors. False positives resulting from cytodiagnosis are actually obtained in routine clinical examinations that often cause problems in later diagnoses and difficulty in dealing with patients. Positive results for CA19–9 and CEA are as low as 10–20% [23, 28], and thus, there are no tumor markers that can be called specific to tumors in urinary tract epithelium.

In recent years, cancers have been recognized as diseases that are induced by gene abnormality or accumulation of chromosomal abnormalities and molecular biological examinations such as detection of gene mutations by the microsatellite method [22,30], detection of mutant CD44 expression by the RT-PCR method [19] and telomerase activity measurement [4,18,32] have been actively performed. The fluorescence in situ hybridization (FISH) method) is a differential staining method for chromosomes developed by Pinkel et al. in 1986 [26]. In this method, chromosomal regions that are complementary to a specific DNA are labeled with fluorescence by directly hybridizing DNA probes to chromosomal preparations on glass slides. Using the FISH method, Ichikawa et al. [3] reported that in mammary cancer, chromosome 1 polysomy was detected in 85%, chromosome 11 polysomy was detected in 55% and chromosome 17 polysomy was detected in 35% of the chromosomal preparations. Jibiki et al. [15] reported

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that the chromosome 17 monosomy was detected in a significant number of stomach cancer cases, and Herman et al. [11] reported Y chromosomal abnormality. Regarding bladder cancer, deletion of chromosome 9 was reported [14]. Using the FISH method, we investigated the incidence of oncogene c-*myc* in chromosome 8 and oncogene H-*ras* in chromosome 11.

Materials and methods

Materials

There were 27 patients (16 males and 11 females) ranging in age from 39 to 87 years (67.0 ± 10.6 years old) with incipient bladder cancer detected between January 1997 and March 1998. These were diagnosed following transurethral resection of the tumors or punch biopsy. Three cases showed false positives for a tumor on urinary cytodiagnosis but on histological examination were diagnosed as dysplasia; three cases were false positives on urinary cytodiagnosis but on histological examination were diagnosed as cystitis. In seven cases tumors were suspected on cystoscopy but both urinary cytodiagnosis and histological examination did not show malignancy. These cases were used as comparative controls. Normal human peripheral lymphocytes were used as normal controls.

Methods

Sample preparation

The bladder was lavaged twice with 50 ml of physiological saline and 100 ml of lavage fluid was collected. The lavage fluid containing cells was centrifuged at 1200 rpm for 5 min and the supernatant was discarded. To the cell pellet, 5 ml of 0.075 M KCl was added and pipetted, then the solution was incubated at 37 °C for 10 min. Two milliliters of Carnoy's fixative (acetic acid:methanol = 1:3) was added and the cells were fixed at 4 °C for 15 min. After centrifuging at 1200 rpm for 5 min, Carnoy's fixative was exchanged. The cell pellet was mixed with 50 µl of Carnoy's fixative and 10 µl of resuspended cells were dropped on a glass slide and air-dried. This was then incubated at 65 °C for 4 h.

Denaturation of preparations

The preparations were soaked in 70% formaldehyde at 75 °C for 5 min, 70% ethanol at −20 °C for 5 min, then 100% ethanol at room temperature for 5 min.

Preparation of probes

To 8.5 µl formaldehyde, 1.5 µl probes, chromosome 8α-satellite (D8Z2) or chromosome 11α-satellite (D11Z1) (Oncor Co), were added. After heat treatment on a heatblock at 75 °C, the probes were cooled immediately in ice. To the denatured probes, 10 µl hybridization solution (mixture of 15 µl bovine serum albumin (BSA) 20 mg/ml, 30 µl 10 × standard saline citrate (SSC) and 30 µl 50% dextran sulfate) were added. The adjusted probes were mounted on slide preparations and uniformly distributed by covering with parafilm. Then, the slide preparations were placed in a tightly closed box filled with 2 × SSC and hybridized with the probe at 37 °C overnight. Fifty percent formamide solution (25 ml formamide, 25 ml 2 × SSC) was prepared and stored at 37 °C. The slide preparations were soaked in 50% formaldehyde solution at 37 °C for 10 min, 2 × SSC for 10 min, then 1 × SSC for 10 min. Then, the slides were washed with 4 × SSC for 5 min to remove nonspecifically bound probes.

FITC (fluorescein isothiocyanate)-labeled avidin solution was diluted 250-fold with 1% BSA/4 × SSC and 70 µl diluted FITC was mounted on the slides and uniformly distributed by covering with parafilm. Then, the slides were incubated in a tightly closed box filled with 2 × SSC at 37 °C for 45 min. The parafilm was removed and the slides were sequentially soaked with shaking (1 200 rpm) on a rotary shaker in the dark for 5 min in 4 × SSC, 0.1% TritonX-100/4 × SSC, then 4 × SSC.

After the slides were soaked in 2 × SSC and left standing for 5 min, 30 µl counter staining solution (25 µl 0.25 µg/µl propidium iodide, 5 µl paraphenylenediamine) was dropped onto the slides and mounted with a cover glass. Excess solution was aspirated with a paper towel and the slides were sealed with nail polish lacquer and left standing for 15 min. Under a fluorescence microscope, 100 nuclei were counted and when 15% or more nuclei showed a single signal, the specimen was regarded as monosomic. When 20% or more nuclei showed three or more signals, the specimen was regarded as polysomic. These definitions were based on the criteria proposed by Ichikawa et al. [3]. For statistical analysis, the relationships between grades and FISH and between stages and FISH were analyzed using the Mann-Whitney U test, and comparison between urinary cytodiagnosis and the FISH method was analyzed using the sign test.

Results

General patient data and results obtained from urinary cytodiagnosis, the FISH method and histopathological examination are shown in Table 1. Urinary cytodiagnosis was performed at least three times and the highest value obtained was used as the result. The results of examining chromosomes 8 and 11 in normal control lymphocytes by the FISH method are shown in Fig. 1. Since a pair of chromosomes is present in human nuclei, two signals are detected in a normal cell nucleus. Figure 2 shows typical chromosomal abnormalities detected by the FISH method. Three signals from chromosome 8 were detected in Case 24 (histology: CIS) and four signals from chromosome 11 were detected in Case 22 (histology: TCC, G3, pT1b). There were no cases showing monosomy.

Of 27 patients histopathologically diagnosed as having malignant bladder tumor (including one case of small cell carcinoma), abnormality in chromosome 8 was detected in 15 patients (55.6%) and 10 of these patients (37.0%) also showed abnormality in chromosome 11. Both chromosomes were normal in 12 patients (44.4%).

Of the three cases that were diagnosed as dysplasia on histological examination but false positive for tumor on urinary cytodiagnosis, one case was negative for both chromosomes 8 and 11 on FISH, one case was positive for chromosome 8 and negative for chromosome 11, and one case was positive for both chromosomes. In three cases that were diagnosed as cystitis by histological examination but false positive for tumors by urinary cytodiagnosis, all cases were negative for both chromosomes 8 and 11 on FISH. Seven cases where the histology did not show malignancy were also negative for both chromosomes 8 and 11 (Table 2). Two dysplasia cases that were positive for one of the chromosomes by the FISH method underwent intravesical

Table 1 General data and results of cytology, FISH and histology

	Sex	Age	Cytology	FISH 8	FISH 11	Histology
1	F	64	Negative	−	−	TCC, G1, pTa
2	F	55	Negative	−	−	TCC, G1, pTa
3	F	60	Negative	−	−	TCC, G1, pTa
4	M	63	Negative	+	−	TCC, G1, pTa
5	M	73	Negative	−	−	TCC, G1, pT1a
6	M	64	Negative	+	−	TCC, G1, pT1a
7	M	63	Negative	+	−	TCC, G1, pT2
8	M	39	Negative	−	−	TCC¤G2, pTa
9	F	87	Negative	−	−	TCC¤G2, pTa
10	M	84	Negative	−	−	TCC, G2, pTa
11	F	68	False positive	+	+	TCC, G2, pTa
12	F	70	False positive	+	+	TCC, G2, pTa
13	M	85	False positive	+	+	TCC, G2, pTa
14	M	71	Positive	+	+	TCC, G2, pTa
15	M	58	Negative	+	+	TCC, G2, pT1a
16	M	72	False positive	+	−	TCC, G2, pT1b
17	M	69	Positive	−	−	TCC, G2, pT2
18	F	77	False positive	−	−	TCC, G3
19	M	65	Positive	−	−	TCC, G3
20	M	54	Negative	−	−	TCC, G3
21	F	55	Positive	−	−	TCC, G3, pT1a
22	F	68	Positive	+	+	TCC, G3, pT1b
23	M	67	Positive	+	+	TCC, G3, pT2
24	M	81	Positive	+	+	CIS
25	M	72	Positive	+	+	CIS
26	F	68	Positive	+	+	CIS
27	F	57	Positive	+	−	Small cell ca
28	F	61	False positive	+	+	Dysplasia
29	F	70	False positive	+	−	Dysplasia
30	M	72	False positive	−	−	Dysplasia
31	F	60	False positive	−	−	Cystitis
32	F	57	False positive	−	−	Cystitis
33	F	37	False positive	−	−	Cystitis
34	F	49	Negative	−	−	No malignancy
35	M	62	Negative	−	−	No malignancy
36	M	67	Negative	−	−	No malignancy
37	M	74	Negative	−	−	No malignancy
38	F	42	Negative	−	−	No malignancy
39	F	55	Negative	−	−	No malignancy
40	M	67	Negative	−	−	No malignancy

infusion therapy. The disease recurred after 6 months in one of these two patients who was positive for chromosome 8 and negative for chromosome 11 and histopathological atypism was diagnosed as grade 3.

Correlation between grades and abnormality in the number of chromosomes was investigated in three CIS cases and 23 cases of bladder cancer excluding one case of small cell carcinoma. In cases diagnosed as grade 1, three out of seven cases (42.9%) were positive for chromosome 8: in cases diagnosed as grade 2, six out of ten cases (60.0%) were positive and in cases diagnosed as grade 3, two out of six cases (33.3%) were positive, showing no correlation between grades and an abnormal number of chromosome 8. Regarding chromosome 11, all cases showing grade 1 were negative, five out of ten cases showing grade 2 were positive (50.0%) and two out of six cases showing grade 3 (33.3%) were positive, also showing no correlation (Table 3).

Correlation with the stage was investigated in 23 cases. Regarding chromosome 8, three out of three were CIS cases (100%), five out of 11 cases were in stage Ta (45.5%), four out of six cases in T1 (66.7%) and two out of three cases in T2 (66.7%) were positive, showing no correlation. With regard to chromosome 11, three out of three were CIS cases (100%), four out of 11 cases in stage Ta (36.4%), three out of six cases in T1 (50.0%): one out of three cases in T2 (33.3%) were positive, showing no correlation (Table 4). The disease relapsed in Cases 1, 6, 10 and 12. Of these recurrent cases, two cases (Cases 6 and 12) (50%) were positive for chromosome 8 and one case (Case 12) (25%) was positive for chromosome 11; among the other 23 cases in which the disease did not relapse, 13 cases (56.5%) were positive for chromosome 8 and nine cases (39.1%) were positive for chromosome 11, showing no significant difference between the presence or absence of relapse and the results of FISH.

Regarding gender difference, ten out of 16 males (62.5%) were positive for chromosome 8, while five out of 11 females (45.5%) were positive, which did not significantly differ. Similarly, there was no significant gender difference detected for chromosome 11 with six of 16 males (37.5%) showing positive and four out of 11 females (36.4%). Regarding age difference, the average

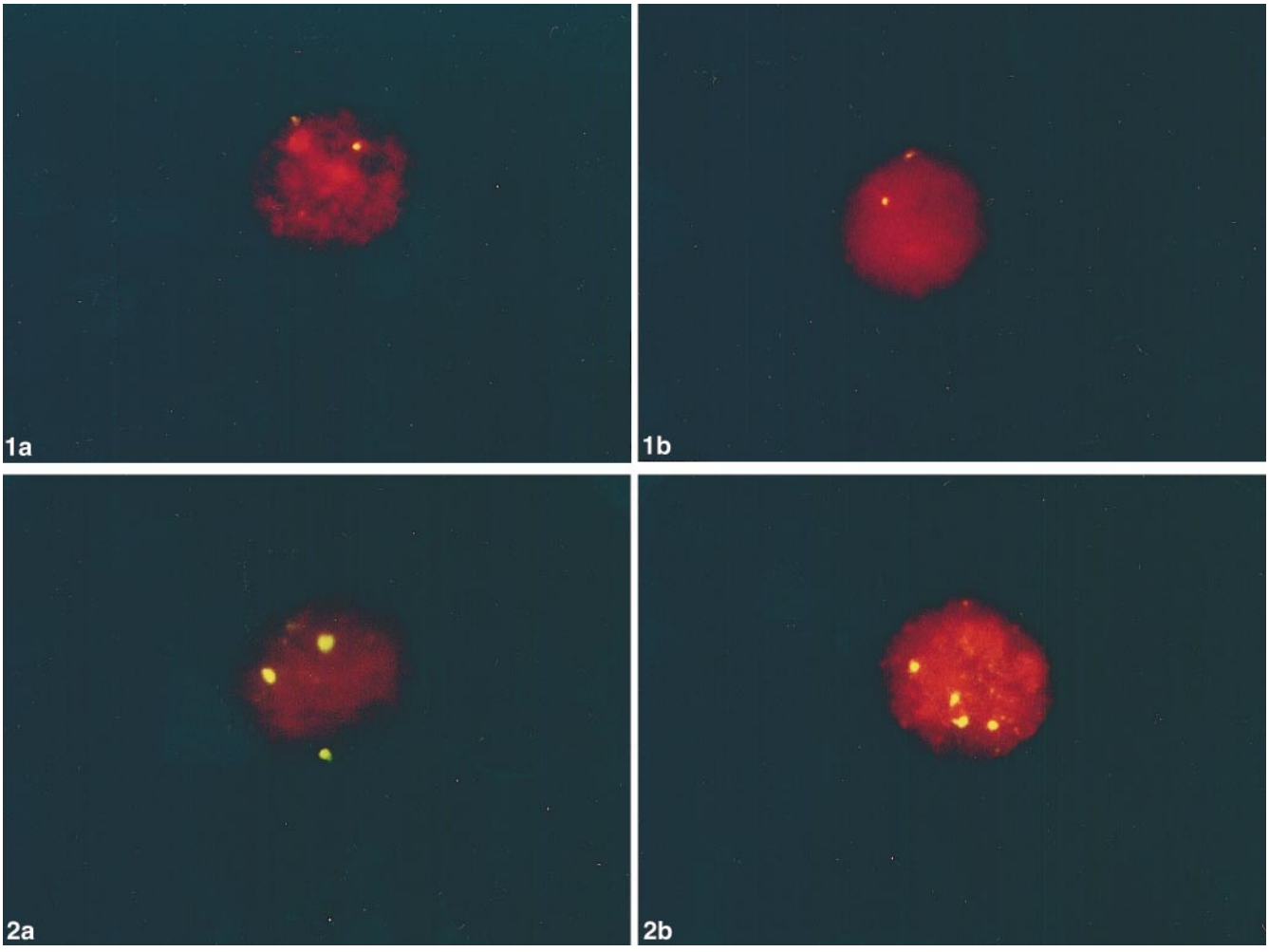

Fig. 1a, b Photomicrograph of fluorescence in situ hybridization on control (lymphocyte) with a biotinylated repetitive probe for chromosome 8 and chromosome 11. Hybridized probes were visualized with fluorescein isothiocyanate (FITC–avidin (*yellow*) nuclei were counterstained with propidium iodide (*red*). **a** Chromosome 8; **b** chromosome 11

Fig. 2a, b Photomicrographs of fluorescence in situ hybridization on bladder tumor cells with a biotinylated repetitive probe for chromosome 8 and chromosome 11. Hybridized probes were visualized with FITC–avidin (*yellow*). Nuclei were counterstained with propidium iodide (*red*). **a** Case 24 showing trisomy for chromosome 8; **b** case 22 showing tetrasomy for chromosome 11

age of patients who were positive for chromosome 8 was 68.5 years, while that for negative patients was 65.1 years, showing no significant difference. Similarly, there was no significant age difference detected for chromosome 11 with average ages of 70.8 years in positive patients and 64.8 years in negative patients.

Of ten cases diagnosed as positive by urinary cytodiagnosis, seven (70%) and six cases (60%) were positive for chromosome 8 and chromosome 11, respectively. Of five cases diagnosed as false positive by urinary cytodiagnosis, four (80%) and three cases (30%) were positive for chromosomes 8 and 11, respectively. Of 12 cases diagnosed as negative by urinary cytodiagnosis, four cases (33.3%) and one case (8%) were positive for chromosomes 8 and 11, respectively. These results showed a correlation with urinary cytodiagnoses ($P < 0.05$). The sensitivities were 55.6% in urinary cytodiagnosis, 55.6% in FISH of chromosome 8 and 37% in FISH of chromosome 11, showing no significant difference. The specificities were 53.8% in urinary

Table 2 Seven cases where the histology did not show malignancy were also negative for both chromosomes 8 and 11 [*FISH* fluorescent in situ hybridization, *Chr* chromosome, (−) disomy, (+) aneusomy]

FISH results (Chr8/Chr11)	Cystitis ($n = 3$)	No malignancy ($n = 7$)	Dysplasia ($n = 3$)	Bladder tumors ($n = 27$)
(−/−)	3	7	1	12
(−/+)	0	0	0	0
(+/−)	0	0	1	5
(+/+)	0	0	1	10

Table 3 Relation between FISH results and grade of bladder tumor (*FISH* fluorescent in situ hybridization, *NS* not significant, *G* grade)

FISH results	G1	G2	G3	Total
Chromosome 8				
Disomy	4	4	4	12 (52.2%)
Monosomy	0	0	0	0 (0%)
Polysomy	3	6	2	11 (47.8%)
Chromosome 11				
Disomy	7	5	4	16 (69.6%)
Monosomy	0	0	0	0 (0%)
Polysomy	0	5	2	7 (30.4%)

Table 4 Relationship between FISH results and stage of bladder tumors. *FISH* fluorescent in situ hybridization, *NS* not significant, *Tis* carcinoma in situ

FISH results	Tis	Ta	T1	T2	Total
Chromosome 8					
Disomy	0	6	2	1	9 (39.1%)
Monosomy	0	0	0	0	0 (0%)
Polysomy	3	5	4	2	14 (60.9%)
Chromosome 11					
Disomy	0	7	3	2	12 (52.1%)
Monosomy	0	0	0	0	0 (0%)
Polysomy	3	4	3	1	11 (47.9%)

cytodiagnosis, 84.6% in FISH of chromosome 8 and 92.3% in FISH of chromosome 11, showing no significant difference.

Discussion

The FISH method is a differential staining method for chromosomes developed by Pinkel et al. in 1986 [26]. In this method, complementary chromosomal regions are visualized using fluorescence-labeled probes for nucleotide sequences of DNA in sample preparations. An advantage of this method, is that it is objective as with morphological observation of cells since chromosomal abnormalities are detected as a number of signals. Furthermore, facilities for handling radioactive substances are not required since probes are labeled with non-radioactive fluorescence, and cell morphology can be simultaneously analyzed using a fluorescent microscope. In a solid tumor C it is difficult to analyze cells at the mitotic phase but not during the intermitotic period [1, 5, 6, 9, 12, 13, 29, 31, 33].

FISH methods can be divided into the following three groups by probes used: (1) methods using chromosome-specific repetitive probes, (2) methods using whole painting probes, and (3) methods using locus-specific probes. In method one, probes for chromosome-specific repetitive sequences located close to the centromere are used and FISH analysis using a painting probe is usually performed in cells in the mitotic period. Its usefulness is limited to analysis of cells in the intermitotic period. In method two, probes entirely covering a chromosome from the end of the short arm to the end of long arm are used and this method may be appropriate for detecting small chromosomal abnormalities using cells in the mitotic period that are difficult to detect by differential staining of chromosomes, and for elucidating fragment origins in marker chromosomes. Method three is the FISH method using probes specific to the locus of oncogenes and tumor suppresser genes, and this method may be useful for investigating amplification and deletion of these genes. In this investigation of tumor cells in the bladder, we focused on chromosomes 8 and 11. It is known that oncogenes c-*myc* and H-*ras* are located on chromosomes 8 and 11, respectively. Abnormalities of c-*myc* and H-*ras* such as mutation, deletion and amplification have been reported for various cancer tissues. It is known that c-*myc* is involved in proliferation, differentiation and apoptosis of cells by regulating expression of various genes as a transcriptional factor, and gene abnormalities in c-*myc* were reported for mammary cancer, lung carcinoma, Burkitt's lymphoma and cancers in the head and neck. Furthermore, association of c-*myc* with grades and stages of bladder cancer was suggested [10]. Ras is known to present on the cell membrane and plays an important role in signaling the regulation of cell proliferation and differentiation. Mutation in the *ras* oncogene has been reported for various cancers including cancers of the pancreas and large intestine, and it is known that the frequency of H-*ras* mutation is especially high in bladder cancer [2,16,25].

We considered that analyzing the abnormalities in chromosomes 8 and 11, where it has been suggested that genes associated with bladder cancer are located, would provide useful information. Therefore, we investigated using a method that differentiated between chromosomes.

Of 27 patients histopathologically diagnosed as having malignant tumors in the bladder, an abnormal chromosome 8 was detected in 15 patients (55.6%; zero cases of monosomy and 15 cases of polysomy). Of these 15 patients, an abnormal chromosome 11 was also detected in 10 patients (37%; zero cases of monosomy and 10 cases of polysomy). Both chromosomes were normal in 12 patients. There was no correlation between the number of the abnormal chromosomes and grade or stage. Guido et al. reported that chromosome 9 was deleted in 28% of cases without correlation with grades or stages.

We encounter patients in routine clinical practice in whom diagnosis by urinary cytodiagnosis, cystoscopy and histopathological examination is difficult and it is difficult to select methods for observing the disease course and effect of therapy. Such cases are expected to be overcome by supplementing these methods with the FISH method; for example, when a case is histopathologically diagnosed as dysplasia and is positive by the FISH method, intravesical infusion therapy should be considered.

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